AD_____

Award Number: DAMD17-99-1-9092

TITLE: Cloning and Charcterization of Active Egr-1 Target Genes by In Vivo Crosslinking

PRINCIPAL INVESTIGATOR: Ian de Belle, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

REPORT DATE: May 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2000	Annual Summary (1 May 99 - 30 Apr 00)

4. TITLE AND SUBTITLE
Cloning and Charcterization of Active Egr-1 Target Genes by In Vivo Crosslinking

5. FUNDING NUMBERS
DAMD17-99-1-9092

6. AUTHOR(S)
Ian de Belle, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Burnham Institute
La Jolla, California 92037

E-MAIL:
idebelle@burnham-inst.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The purpose of this project is to identify the profile of Egr-1 target genes in breast cells. Normal breast cells are shown to express Egr-1, while breast cancer cells do not. It is, therefore, important to identify the nature of those target genes regulated by Egr-1 which are absent in breast cancer cells. I have approached this goal by performing in vivo crosslinking of Egr-1 to its target sites in breast cells, followed by immunocapture of Egr-1 together with its targets. In this report, I have confirmed expression of Egr-1 in normal, but not in breast cancer cells. Furthermore, I have succeeded in capturing Egr-1 and its target DNA sites by immunoprecipitation. In addition, I have proceeded by cloning the Egr-1 target DNAs for further characterization. Significantly, I have also developed a technique of multiplex PCR using the captured DNA as primers to identify those Egr-1 binding sites adjacent to, and possibly regulating, genes from a cDNA library. This technique will be used to focus on Egr-1 targets which affect gene expression.

14. SUBJECT TERMS
Breast Cancer, crosslinking, transcription factors, target gene identification

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

MB Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

[signature] 5/30/00

PI - Signature Date

Table of Contents

Introduction

This report provides a detailed description of my accomplishments for the first year of funding supported by the USAMRMC Breast Cancer Research Program. The subject of my research is to identify and clone target genes for the transcription factor Egr-1. The purpose is to gain an understanding of the profile of genetic targets for Egr-1 in normal breast cells which are absent from breast cancer cells. The long term goal is to build transcriptional profiles for Egr-1 and to identifying key transcriptional defects occurring in breast cancer cells. For this report, the scope of the research is to first confirm the expression of Egr-1 in normal, but not in breast cancer cells. Subsequently, Egr-1 must be crosslinked to its target sites *in vivo* through the action of a buffered formaldehyde solution, and the crosslinked Egr-1, together with its bound DNA, isolated for further characterization.

Body

The first task accomplished for this project was to confirm the expression of Egr-1 in MCF10A cells, but not in MCF7 cells. This result confirmed that Egr-1 is expressed in normal, but not in breast cancer cell types. (Figure 1).

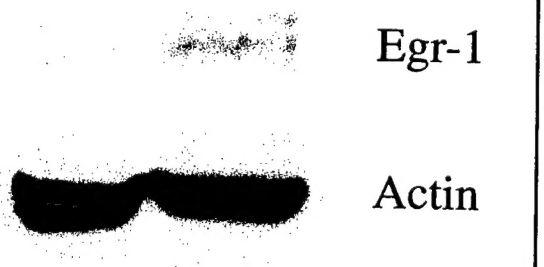

Figure 1. Expression of Egr-1 in MCF10A, but not in MCF7 cells.

After having confirmed expression of Egr-1 in normal breast cells, the next goal was to achieve formaldehyde-mediated crosslinking of Egr-1 to its target binding sites. Once crosslinking was achieved, Egr-1 together with its bound DNA was immunoprecipitated from the purified chromatin fraction. Figure 2 shows the specific immunoprecipitation of crosslinked Egr-1 using an affinity purified antibody prepared in this laboratory.

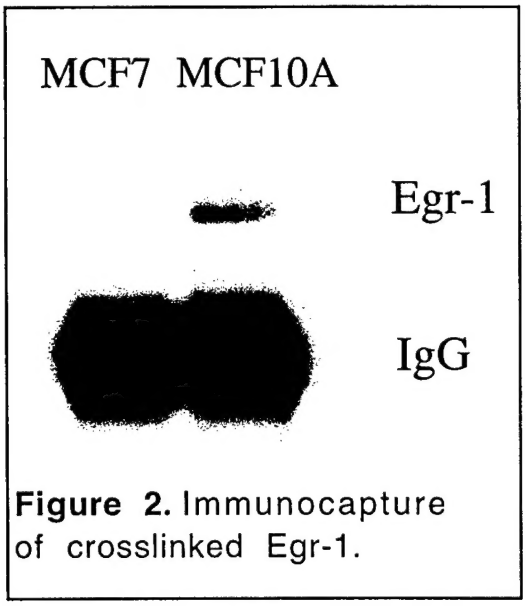

Figure 2. Immunocapture of crosslinked Egr-1.

Once Egr-1 and its crosslinked target sites were captured, the next task was to perform linker ligation onto the captured DNA targets followed by their amplification. This was done in order to obtain sufficient DNA quantity to allow further characterization. Figure 3 shows the specific amplification of DNA target sites from Egr-1 immunoprecipitates from MCF10A, but not from MCF7 cells.

This result highlights the successful use of the *in vivo* crosslinking technique to recover DNA targets directly bound by a transcription factor, in this case Egr-1. These target DNA sites represent regulatory sequences through which Egr-1 exerts its effects in normal, but not abnormal, breast cells. It is these sequence, therefore, that are to be characterized in further detail in order to put together a profile of Egr-1 dependent transcriptional events. To this end, all of these DNA targets have been cloned into a vector for their propagation, and large scale DNA preparations have been done. All of these captured DNA targets are, therefore, available for further characterization.

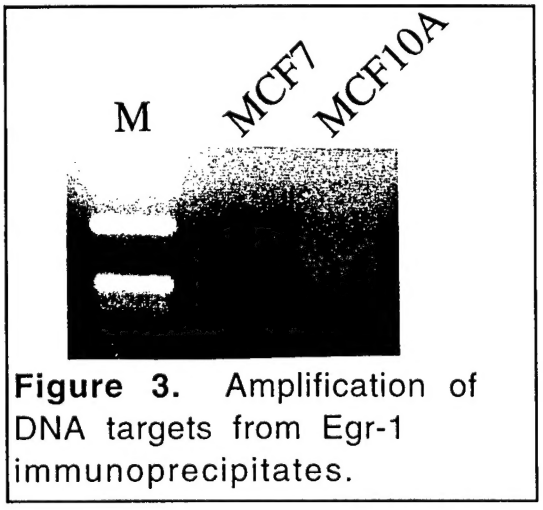

Figure 3. Amplification of DNA targets from Egr-1 immunoprecipitates.

The cloning of Egr-1 target DNA sites represents a major goal in this project, since all of the Egr-1 targets have been isolated from the bulk of genomic DNA. It remains, however, the complex task of identifying from amongst these cloned targets those target sites through which key gene regulation effects are accomplished. A direct screening of binding sites can be performed at this stage to ensure specificity of Egr-1 binding followed by sequencing to identify the target site. Since this approach would

require the individual sequence characterization of hundreds of isolated sequences, it was decided to address the issue of focusing of functionally relevant sequences within the library of Egr-1 targets. To this end, having achieved the cloning of a "library" of Egr-1 target sites, the next goal centered on identifying, from amongst the many binding sites present in the library, those which are functionally relevant to transcriptional regulation by Egr-1

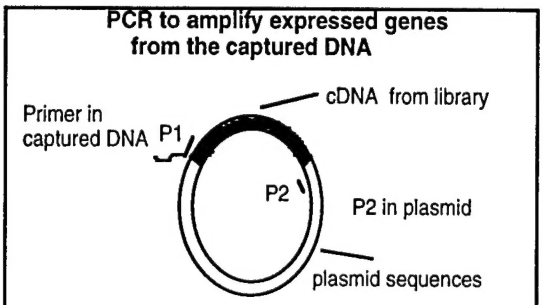

Figure 4. Multiplex PCR strategy.

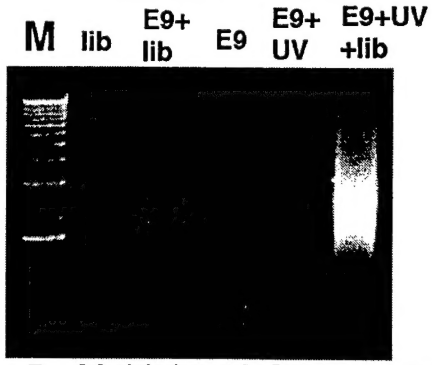

Figure 5. Multiplex PCR amplification of captured Egr-1 targets from a cDNA library. E9 cells express Egr-1, and are activated by UV exposure.

in breast cells. To accomplish this, I have developed a method to isolate from the library of sequences, those which consist of regulatory sequences adjacent to expressed genes. The method consists of multiplex PCR amplification from a breast cDNA library, and is depicted in figure 4. The method was developed using fibrosarcoma cells either deficient in, or overexpressing by stable transfection, Egr-1. It can be seen in figure 5 that the amplification of expressed cDNA sequences can be achieved using this methodology. I am now in the process of applying this technique to the amplification of Egr-1 targets derived from MCF10A cells. By incorporating this application, I will be able to eliminate those target sites which do not contribute to transcriptional regulation by Egr-1, and focus solely on those which represent target sites capable of gene regulation. In this way, I am proceeding to build a profile of Egr-1 target genes in breast cells which will contribute

to an understanding of the aberrant genetic events occurring in breast cancer cells.

Appendix

1) Key research accomplishments:

* Confirmed expression of Egr-1 in normal (MCF10A), but not in breast cancer cells (MCF7).

* Successfully crosslinked Egr-1 to its target sites *in vivo* in MCF10A cells. Successfully captured Egr-1 together with target DNA from these cells.

* Amplified Egr-1 bound targets from immunoprecipitates, and cloned targets into a vector to provide a library of Egr-1 bound DNAs for further characterization.

* Developed a method of multiplex PCR to allow the further characterization of functionally significant DNA target sites from within the library of targets. This method allows the identification of target sites which lie adjacent to expressed cDNAs, and amplifies the cDNAs themselves for identification.

2) Reportable outcomes:

Manuscript: I. de Belle, D. Mercola, and E.D. Adamson (2000). A method for cloning *in vivo* targets of the Egr-1 transcription factor. *Biotechniques* (In press, July, 2000).

Funding applied for: USAMRMC Prostate Cancer Research Program. New Investigator Award. Cloning and characterization of Egr-1 target genes in prostate cancer. Proposed start date: October 2000.

A method for cloning *in vivo* targets of the Egr-1 transcription factor

Ian de Belle [1]*, Dan Mercola[2] and Eileen D. Adamson[1]

[1]The Burnham Institute, 10901 N. Torrey Pines Rd., La Jolla, CA 92037

[2]Sidney Kimmel Cancer Center, San Diego, CA 92121

* To whom correspondence should be addressed.
Tel.(858) 646-3100
FAX (858) 646-3195
e-mail: idebelle@burnham-inst.org

KEYWORDS: Formaldehyde, crosslinking, gel shifts, transcriptional regulation, TGFβ1

ABSTRACT

A methodology is described that allows the *in vivo* trapping of transcription factors to their target regulatory elements in multiple genes simultaneously. Cross-linking using formaldehyde is the first of several steps to isolate, purify, clone and characterize multiple gene promoter DNA fragments. The example that we use indicates that the TGFβ1 gene is a direct target induced by Egr-1 in HT1080 cells that express constitutive Egr-1, thus explaining the growth retardation that follows Egr-1 expression. The genes identified using this procedure reflect the specific activities of Egr-1 at that moment in the cell and provide a method for confirmation of genes that are the direct targets of Egr-1 action.

MATERIALS and METHODS

Cells and culture conditions

The human fibrosarcoma HT1080 cell clone, H4, was kindly provided by Dr. S. Frisch (The Burnham Institute). H4 cells stably expressing Egr-1, E9 cells, have been previously described (9). Cells were cultured in DMEM containing 10% fetal bovine serum and maintained in a 5% CO_2 atmosphere at 37°C.

Chemical crosslinking and chromatin isolation

Formaldehyde induced crosslinking and chromatin isolation was performed as previously described (11, 3). An outline of the procedure is shown as a flow diagram in Figure 1. The optimal time of exposure of H4 and E9 cells to formaldehyde was determined by pilot experiments to be 1-2 hours. This length of exposure was found to give the best yield of Egr-1 capture from the crosslinked chromatin. Crosslinking was performed on approximately 1 X 10^7 attached cells in 15 cm dishes. Chromatin, purified by urea gradient centrifugation, was stored at -80°C. Prior to immunoprecipitation, 30-60 µg of DNA in chromatin was digested overnight at 37°C with 60 U of EcoRI.

Antibodies and immunoprecipitation

An affinity purified rabbit polyclonal antibody (8) raised against amino acids 27-318 was used for both immunoprecipitation of and Western blotting for Egr-1. The antiserum was affinity purified over a column of recombinant Egr-1 coupled to CNBr-activated Sepharose beads.

For immunoprecipitation, 30-60 µg of crosslinked, and EcoR1 digested chromatin was brought to a volume of 1 ml in RIPA buffer to which 1µg of affinity purified anti-Egr-1 antibody was added together with 40 µl of a 50% suspension of protein A-Sepharose beads. Samples were rotated overnight at 4°C, and the bead-captured immunecomplexes

were washed 4 times with RIPA buffer. Samples were then washed with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and divided into two equal aliquots.

For Western blotting, one aliquot was washed with 10 mM Tris, pH7.5, 2 mM $MgCl_2$ and then resuspended in 100 µl of the same buffer with 10 U DNase I and incubated at 37°C for 10 min. The samples were then boiled in SDS sample buffer for 10 min and analyzed by 7.5% SDS-PAGE. Western blotting was performed using the same anti-Egr-1 affinity purified antibody at a 1:1,000 dilution.

For DNA recovery from the second aliquot, crosslinks were reversed with heat and proteinase K digestion, and DNA purified as previously described (3). Using both proteinase K digestion and heat treatment has been shown to achieve total reversal of crosslinking yielding completely protein-free DNA (15).

Linker ligation and PCR amplification of captured DNA

The purified DNA following crosslink reversal was re-digested with 20 U EcoRI at 37°C for 2 hours to ensure all ends were compatible with the linkers. The digested DNA was ligated to EcoRI linkers consisting of the following oligonucleotides: 5' AATTCGAAGCTTGGATCCGAGCAG 3', and 5' CTGCTCGGATCCAAGCTTCG 3'. Following linker ligation at 16°C overnight, the ligated DNA was subjected to PCR amplification.

PCR amplification of the linker ligated DNA was performed using the 20mer oligonucleotide from the linker ligation as the PCR primer and Pfu polymerase (Stratagene, San Diego, CA), using buffer conditions supplied by the manufacturer. PCR cycling parameters were as follows. 95°C/45 sec., 65°C/30 sec., 72°C/5 min. The number of cycles was determined empirically such that little or no PCR products were visible in the

strong inducer of Egr-1. However, when H4 cells were stably transfected with Egr-1, several clones expressing Egr-1 robustly in a constitutive manner were isolated and one of these was named E9 (9). Since these cells provide an ideal comparison for the effects of Egr-1 expression, we chose for the present study to characterize the binding of Egr-1 to its *in vivo* sites in E9 cells. To assess the immunocapture of Egr-1 to its binding sites we performed Western blotting of Egr-1 following immunoprecipitation of crosslinked and digested chromatin from both H4 and E9 cells. Since Egr-1 can be induced and activated by a variety of extracellular stimuli, we also examined Egr-1 immunoprecipitates from cells treated with 40 J/m^2 of UV-C and incubated for a period of 2 hours following irradiation. Figure 2 shows the results of Egr-1 immunoprecipitation from samples of crosslinked chromatin. In H4 cells there was little or no Egr-1 present in the immunoprecipitates as expected. In the Egr-1 constitutively expressing E9 cells, however, we successfully immunoprecipitated Egr-1 from both untreated and UV-C treated cells. These results indicate that at least some of the Egr-1 expressed in E9 cells is bound to DNA *in vivo* and therefore has the potential to be functional. Furthermore, we observed that compared to untreated E9 cells, following UV-C irradiation our immunoprecipitates contained considerably more Egr-1, suggesting that this stimulus promoted an increase in the binding of Egr-1 to its target sequences (Fig. 2, compare lanes 3 and 4).

Amplification of Egr-1 bound DNA

To assess the capture of Egr-1 bound DNA sequences we performed linker ligation followed by PCR amplification on samples of Egr-1 immunoprecipitates from H4 and E9 cells. Figure 3 shows that after 20 cycles, DNA was amplified from E9, but not from H4 immunoprecipitiates. DNA fragments ranging in size from 0.5-3 kb were detected. This result is consistent with the presence of Egr-1 in E9, but not in H4 chromatin immunoprecipitates shown by Western blotting (Fig. 2), and is a direct demonstration that Egr-1 is bound to DNA in these cells. Our results also suggest that following UV

irradiation there may be additional sites to which Egr-1 binds as evidenced by a different amplification profile seen in irradiated E9 cell (compare Fig. 3, lanes 3 and 4).

The expression of Egr-1 in E9 cells is constitutive, and it is possible that the level is higher than one could expect after the normal induction of Egr-1. Therefore, we also tested physiologically-induced Egr-1 expression in MCF7 human mammary carcinoma cells, using a two hour exposure to tetradecanoylphorbol acetate, TPA. On a Western blot, the level of Egr-1 protein was two-fold higher than that expressed in E9 cells indicating that the method does not require high constitutive Egr-1 expression (data not shown). Also, multiple DNA fragments were captured using crosslinking of the Egr-1 to its target genes in MCF7 cells following our method. The sensitivity of the method is likely determined by the avidity of the antibody. To test the generality of the method, we also applied antibodies to c-Jun, with appropriate controls, with the result that presumptive c-Jun target genes were also captured after cross-linking (data not shown).

Specific binding of Egr-1 to captured and cloned binding sites

To demonstrate that the DNA sequences which were amplified from E9 cells represent specific target binding sites for Egr-1, we performed gel shift assays on individual captured binding sites. Individual DNA sequences were isolated by ligating PCR products into a cloning vector and selecting single bacterial colonies after transformation as described in the Materials and Methods section. Figure 4A presents gel shift results from three individually cloned and isolated Egr-1 binding sites from E9 cells. Purified recombinant Egr-1 bound to each of these Egr-1 binding sites (EBS). Egr-1 binding was competed off by unlabeled wild type consensus oligonucleotides, but was unaffected by the mutant oligonucleotides, indicating that the binding was specific. The specific binding of Egr-1 demonstrated here confirms that our method is effective in selecting for DNA sequences to which a transcription factor, in this case Egr-1, is directly and specifically bound in the cell.

and has the potential to influence the transcriptional activity at these sites simultaneously. While we have presented results on three individual cloned DNA sequences, these are presented as examples and we have generated, by this method, an Egr-1 binding site "library" which is being studied further.

It is possible that formaldehyde could crosslink protein to DNA nonspecifically, and it is likely that not all of the genuine binding sites are active in regulating transcription under all conditions. The cloning of transcription factor binding sites by this method must therefore include characterization for specificity of binding and a demonstration of functional activity associated with the binding of the transcription factor to the DNA. During the characterization of binding sites, some obvious considerations include the requirement for heterodimerization or cofactors. Dimerization has not been observed in the case of Egr-1, however. In many cases a single transcription factor does not trans-regulate alone, but rather contributes to gene regulation as part of a complex set of protein/DNA and protein/protein interactions.

Despite these considerations we have, for the first time, successfully cloned DNA sequences which both bind Egr-1 directly and influence transcription. In one case presented here, we have cloned a sequence which not only binds Egr-1, but is also UV responsive (Fig. 4B, pEBS-3), demonstrating the possibility of identifying specific targets of transcription factors in response to a specific stimulus. In another example, we showed that TGFβ1 gene promoter sequences were captured by crosslinking to Egr-1 in E9 cells (Fig. 5). This not only proves the principle of the method (because TGFβ1 is a known target of Egr-1 induction), but also is the first demonstration that Egr-1 functions *in vivo* by its *direct* binding to the TGFβ1 promoter. Our observation that Egr-1 was not bound to the TGFβ1 promoter following UV irradiation may indicate that, following this stimulus, there is a decrease in the Egr-1-induced growth suppression in these cells. This would be

consistent with our recently published results showing that following UV treatment, the expression of Egr-1 is associated with an increase in cell survival which correlates with a transient increase in the rate of cell cycle progression (4).

Moreover, we are developing a further step in this cloning method, by using the longest captured DNA fragments as labeled probes to hybridize to multiplex arrays of cDNAs. Clearly, this technique has potential utility in dissecting the diverse activities of transcription factors responding to a variety of signals and may reveal novel genetic targets of these factors. Moreover, the elucidation of gene clusters that are co-regulated by a stimulus is a major advantage of this method.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support of grants form the Public Health Services, CA 67888 (E.D.A.) and CA 63783 and CA76173 (D.M.) from the National Cancer Institute and DAMD 17-99-9092 (I. de B.) from the Department of Defense.

Captured DNA sequences will be made available upon request.

FIGURE LEGENDS

Figure 1. Flow chart to illustrate the early steps of the DIVET cloning procedure.

Figure 2. Egr-1 protein recovered from crosslinked chromatin. After immunoprecipitation of the chromatin fragments containing Egr-1, the DNA was digested away and the recovered protein was analyzed by SDS-PAGE and immunoblotting with the same affinity purified rabbit anti-Egr-1 IgG. Lanes 1 and 2, show that little if any Egr-1 is recovered from H4 cells before and after UV irradiation. Lanes 3 and 4, Egr-1 recovered from E9 cells indicate a substantial increase in target frequency in UV treated cells.

Figure 3. DNA fragments captured from HT1080 cells analyzed on an agarose gel. DNA fragments were amplified by PCR after reversal of crosslinks between Egr-1 and its target DNA (see Methods for details). The lanes refer to the same cell extracts as in Figure 2.

Figure 4A. Gel Shift assays to show the binding specificity of captured DNA fragments. Three different DNA fragments were labeled and tested for putative Egr-1 binding sites (EBS). Recombinant GST-Egr-1 was allowed to bind as described in the Methods section. Unlabeled wild type Egr-1 binding site oligonucleotides and mutated oligonucleotides were added to the indicated samples following Egr-1 binding as described in the Methods section. In all three cases, only the wild type Egr-1 binding site oligonucleotides competed for the putative EBS. The concentration of Egr-1 used in gel shift studies, shown as 1, 2 and 3 corresponds to 50, 100 and 200 ng of protein, respectively.

4B. Transactivation assays with a luciferase reporter gene. The same captured DNAs as in A, were ligated to a minimal promoter-luciferase gene. After transient

transfection into H4 cells, the luciferase activity of cells co-transfected with Egr-1 (black bars) was induced compared to the "empty-vector" control (white bars). Only EBS3 DNA contained a target promoter that was activated by UV.

Figure 5. The capture of an authentic Egr-1-regulated promoter, the TGFβ1 promoter, in the DNA targets from E9 cells provides proof of principle that DIVET cloning is practical. Primers that bracket the putative Egr-1 binding site in the TGFβ1 promoter were used with the captured DNA as templates in a PCR reaction. A band of the predicted size indicated that the TGFβ1 promoter was captured in the recovered crosslinked DNA.

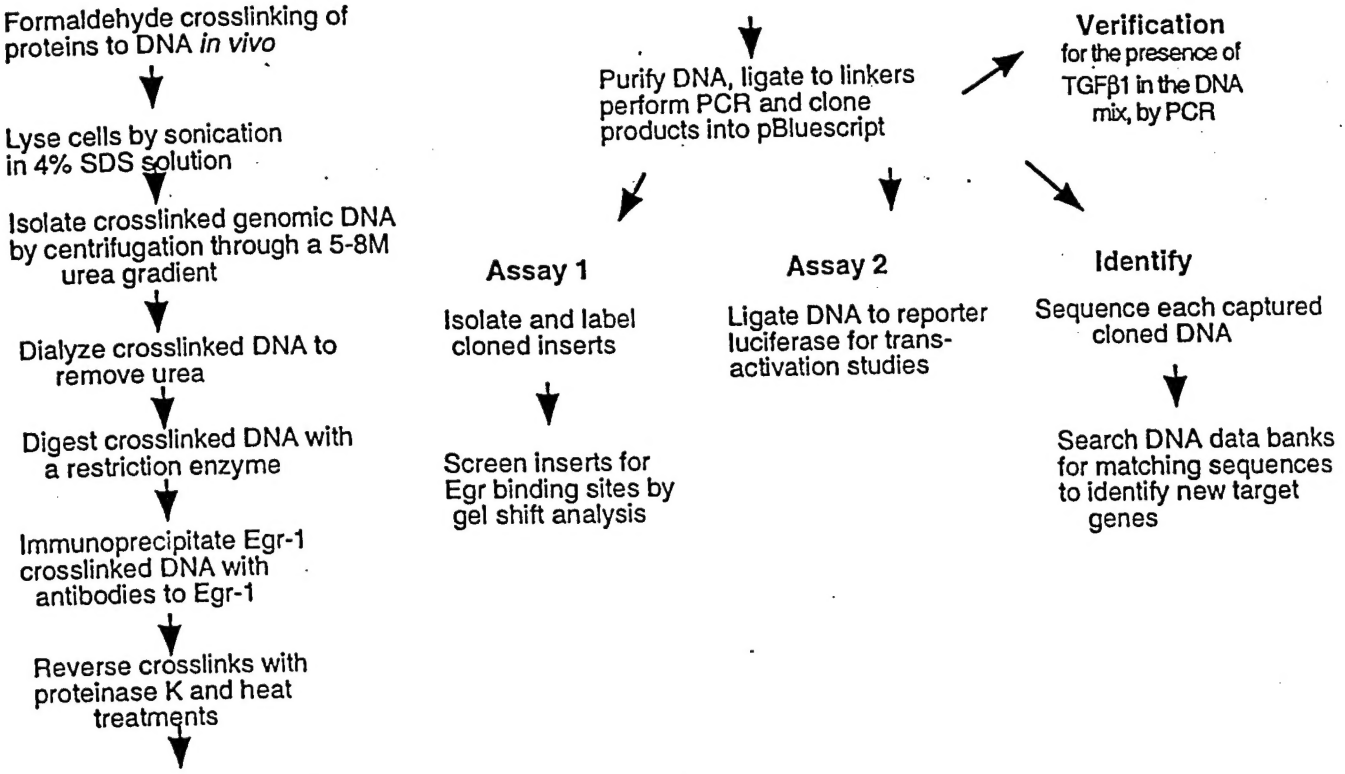

Figure 1 - Flow Chart of steps to clone *in vivo* target genes of Egr-1 (DIVET cloning)

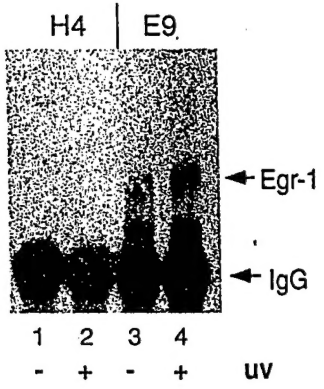

Figure 2

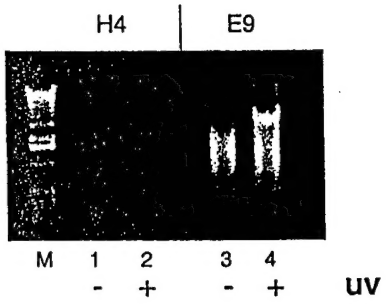

Figure 3

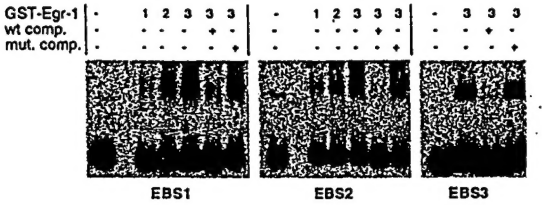

Figure 4A

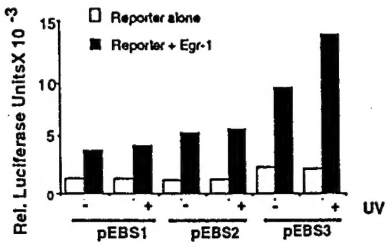

Figure 4B

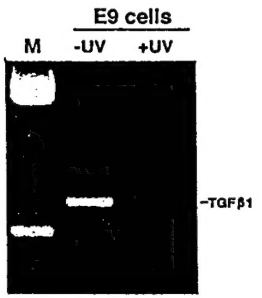

Figure 5